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ORIGINAL ARTICLE

Influence of preoperative anti-cancer therapy on resectability and perioperative outcomes in patients with pancreatic cancer: Project study by the Japanese Society of Hepato-Biliary-Pancreatic Surgery

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Abstract
Background Little is known about the effects of neoadjuvant therapy on outcomes in patients with pancreatic cancer. This study evaluated the effects of neoadjuvant therapy on resectability and perioperative outcomes.
Methods A total of 992 patients were enrolled, with 971 deemed eligible. Of these, 582 had resectable tumors and 389 had borderline resectable tumors, and 388 patients received neoadjuvant therapy. Demographic characteristics and peri- and postoperative parameters were assessed by a questionnaire survey.
Results The R0 rate was significantly higher in patients with resectable tumors who received neoadjuvant therapy than in those who underwent surgery first, but no significant difference was noted in patients with borderline resectable tumors. Operation time was significantly longer and blood loss was significantly greater in patients who received neoadjuvant therapy than in those who underwent surgery first, but there were no significant differences in specific complications and mortality rates. The node positivity rate was significantly lower in the neoadjuvant than in the surgery-first group, indicating that the former had significantly lower stage tumors.
Conclusions Neoadjuvant therapy may not increase the mortality and morbidity rate and may be able to increase the chance for curative resection against resectable tumor.

Keywords Neoadjuvant · Pancreatic cancer · Perioperative outcome · Resectability · Surgery

Introduction

Patients with pancreatic cancer have a dismal prognosis, even when tumors are resectable. Both local and systemic recurrences are common after curative (R0) resection, and long-term survival rates are low. The standard treatment for patients with resectable pancreatic cancer is surgery followed by adjuvant chemotherapy [1–5], but the 2-year postoperative survival rate remains below 50% [3–5].

Neoadjuvant therapy has been used as an alternative approach in other types of cancer, including breast and esophageal cancers. In breast cancer patients, neoadjuvant chemotherapy has been shown to effectively reduce tumor burden in the breast and axilla without compromising survival [6]. In esophageal cancer patients, preoperative chemotherapy was found to result in longer overall survival than postoperative chemotherapy, and therefore, neoadjuvant chemotherapy became the standard treatment strategy for patients with resectable esophageal cancer [7]. Although reports from single institutions and prospective phase II trials found that neoadjuvant treatment had survival benefits in patients with pancreatic cancer [8–11], no large randomized trials have been performed yet to confirm these results.

The neoadjuvant strategy is subject to two major hypothetical risks: (1) possible increases in operative morbidity and mortality; and (2) the possibility that the disease may metastasize or become unresectable during the course of neoadjuvant chemotherapy [12]. The resectability and perioperative outcomes in patients with resectable and borderline resectable pancreatic cancer could not be assessed in prospective trials of adjuvant chemotherapy [3–5] because these trials did not include patients with metastases detected intraoperatively or soon after surgery, patients who died due to surgical complications, and those who experienced severe morbidity and delayed surgical recovery. A survey is required to evaluate the effects of neoadjuvant treatment in patients intended for pancreatic resection.

Therefore, to clarify this situation, the Japanese Society of Hepato-Biliary-Pancreatic Surgery (JSHPBS) surveyed high-volume centers throughout Japan that had experience with neoadjuvant therapy to evaluate the influence of neoadjuvant therapy on resectability and perioperative outcomes.

Patients and methods

A questionnaire was sent to all patients with pancreatic cancer who were scheduled to undergo resection with curative intent between January 2007 and December 2009 at the 17 high-volume centers participating in the JSHPBS study. This study was approved by the institutional review board of Tohoku University.

The eligibility of this study was invasive ductal adenocarcinoma of the pancreas, which was resectable or borderline resectable intending to surgery. Other types of histology were ineligible, such as acinar cell carcinoma, neuroendocrine tumor, cystic neoplasms. The demographic and clinical characteristics evaluated included patient age, gender, body mass index (BMI), comorbid illness, preoperative tumor staging and resectability [13], and pre- and post-treatment levels of tumor markers. Preoperative treatment data included chemotherapeutic agents; whether or not radiation was administered; the planned and administered doses of both; and adverse events (AEs), both hematological and non-hematological, during preoperative treatment, as assessed by Common Terminology Criteria for Adverse Events ver3.0 [14]. Operative findings included macroscopic tumor stage and intraoperative parameters, such as blood loss, duration of operation, and blood transfusion requirements. Pathological findings included pathological staging, residual tumor status, the effect of preoperative treatment, and intraoperative mortality. Postoperative data included postoperative complications such as pancreatic

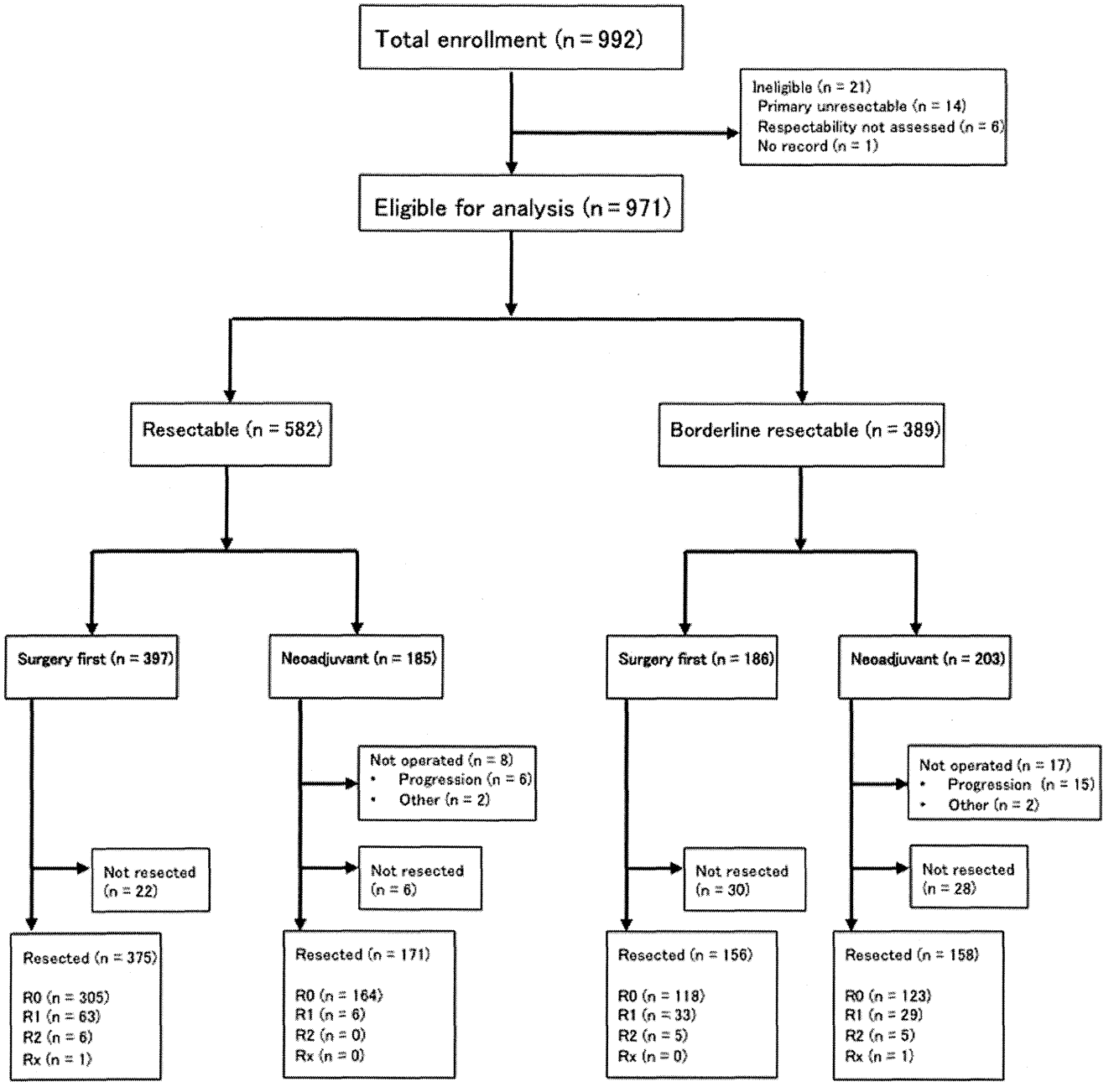

Fig. 1 Flow diagram

fistula, defined according to ISGPF (postoperative pancreatic fistula: an international study group) criteria [15]; delayed gastric emptying, as defined by the ISGPS [16]; other non-abdominal complications; postoperative hospital stay; and types of adjuvant treatment.

Of the 992 patients enrolled, 21 were excluded: 14 with primary unresectable tumors, six who were not assessed for resectablity, and one with no clinical records. Thus, 971 patients were included. Primary outcomes included resectability and perioperative morbidity and mortality. To minimize biases associated with tumor stage, all eligible patients were stratified according to the presence of resectable or borderline resectable tumors, as defined by the National Comprehensive Cancer Network (Fig. 1). The tumor without any abutment of major vessel including portal vein/superior mesenteric vein (PV/SMV), superior mesenteric artery, hepatic artery, celiac artery was categorized in resectable. The tumor with impingement of PV/SMV but reconstructable and/or major arterial abutment within 180 degrees, which was considered to be separable at surgery was categorized as borderline. The indication of resection depended on each institution surveyed.

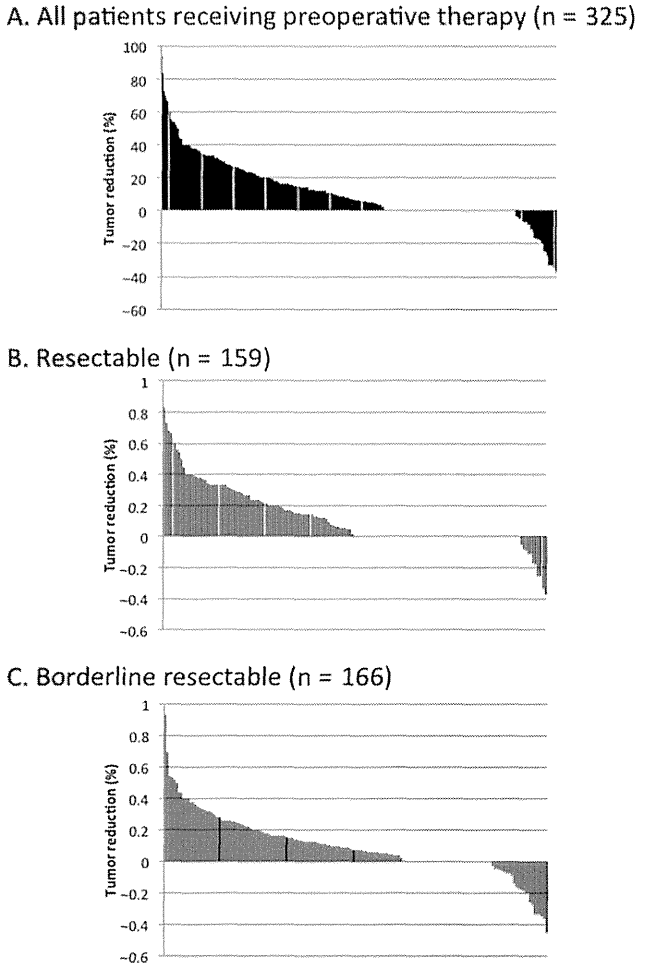

Fig. 2 Radiological tumor response

Assessment of resectability was answered by questionnaire survey. Resectability and R0-resectability were calculated on an intention-to-treat basis, and therefore, patients who did not undergo surgery for any reason were included. Perioperative morbidity and mortality of patients receiving neoadjuvant therapy (neoadjuvant patients) and those undergoing surgery without neoadjuvant therapy (surgery-first patients) were compared separately in subgroups of patients with resectable and borderline resectable tumors, because of differences in operative procedures, such as major vessel resection (Tables 4,5). The efficacy of neoadjuvant therapy could be assessed radiologically in 325 of the 389 patients (83.5%). Best percentage change from baseline in the size of the primary tumor was shown by waterfall plot analysis (Fig. 2).

Statistics

Continuous variables were expressed as median and range. Between group differences in patient characteristics and perioperative and postoperative factors were compared using χ^2 tests, Fisher's exact test, and Mann–Whitney's *U*-test, as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Patient characteristics

Of the 971 included patients, 582 had resectable and 389 had borderline resectable tumors. The clinical characteristics of these patients are shown in Table 1. Patients with borderline resectable tumors were significantly younger ($P < 0.001$) and had jaundice followed by biliary drainage more frequently ($P < 0.001$) than those with resectable disease. Pre-treatment serum concentrations of tumor markers were significantly higher in patients with borderline resectable tumors than in those with resectable tumors. Medical history did not differ significantly, except that previous malignancies were significantly more frequent in the resectable group ($P = 0.012$). In each subgroup, of patients with resectable and borderline resectable tumors, there were no statistically significant differences in age, sex, presence of jaundice, and serum tumor markers between patients who received neoadjuvant treatment and those who underwent surgery first (data not shown).

Neoadjuvant therapy

A total of 388 patients (40%) received neoadjuvant treatment, including 254 who received radiotherapy or chemoradiotherapy and 115 who received systemic chemotherapy. Types of therapy and agents are summarized in Table 2. Neoadjuvant treatment was significantly more common in patients with borderline resectable than in those with resectable cancers (52% vs. 32%, $P < 0.0001$). Gemcitabine or a gemcitabine-based regimen was the most frequently provided for chemoradiotherapy and systemic chemotherapy. In regard to neoadjuvant radiotherapy, the duration of preoperative therapy in the resectable group was significantly longer than that in the borderline group (99.5 days vs. 82 days). Whereas in regard to neoadjuvant chemotherapy, the duration in the resectable group was significantly shorter than that in the borderline group (28 days vs. 81.5 days).

Feasibility and efficacy of neoadjuvant therapy

Hematological and non-hematological AEs during neoadjuvant therapy are shown in Table 3. There were no neoadjuvant therapy-related deaths. Neutropenia and

Table 1 Baseline demographics

	Resectable	Borderline	*P*-value
Number of patients	582	389	–
Age (y), median (range)	68 (27–90)	65 (32–87)	<0.001
Sex (male : female)	320:262	229:160	0.23
Past medical history or Comorbid illness			
Hypertension, *n* (%)	166 (29)	92 (24)	0.091
Coronary arterial disease, *n* (%)	66 (11)	36 (11)	0.30
Diabetes mellitus, *n* (%)	175 (30)	120 (31)	0.80
Other malignancy, *n* (%)	94 (16)	41 (11)	0.012
Peptic ulcer, *n* (%)	45 (7.8)	25 (6.4)	0.44
Hepatitis, *n* (%)	39 (6.7)	20 (5.1)	0.31
Other digestive disease[a], *n* (%)	98 (17)	49 (13)	0.074
Respiratory disease, *n* (%)	41 (7.1)	20 (5.1)	0.22
Cerebral Vascular disease, *n* (%)	25 (4.3)	17 (4.4)	0.96
Jaundice, *n* (%)	190 (33)	169 (43)	<0.001
Biliary drainage, *n* (%)	187 (32)	169 (43)	<0.001
Pre-treatment serum tumor marker			
CEA (ng/ml), median (range)	3 (0–435)	3.5 (0–675)	0.0039
CA19-9 (U/ml), median (range)[b]	78 (0–47,470)	203.75 (0–220,540)	<0.001

[a] Other digestive disease included appendicitis ($n = 47$), cholecystolithiasis ($n = 33$), colonic polyps ($n = 16$), pancreatitis ($n = 10$), gastritis ($n = 8$), gastric polyps ($n = 5$), intestinal obstruction ($n = 4$), liver cirrhosis ($n = 3$), reflux esophagitis ($n = 2$), fatty liver ($n = 2$), hepatic hemangioma ($n = 2$), pancreatic cyst ($n = 2$), hemorrhoid ($n = 2$), irritable bowel disease, trauma, situs inversus, colonic derticulitis, ulcerative colitis, Peutz-Jeghers syndrome ($n = 1$), unknown ($n = 5$)

[b] The values of CA19-9 were measured after biliary drainage when the patients with jaundice

Table 2 Types of neoadjuvant therapy and agents

	Resectable	Borderline	*P*-value
Neoadjuvant therapy, *n* (%)	185 (32)	203 (52)	<0.0001
Types of therapy			
Radiotherapy, *n* (%)	114 (20)	140 (36)	<0.0001
Irradiation (Gy), median (range)	50 (35.2–54)	45 (10.8–67.5)	<0.0001
Agents provided			
With Gemcitabine	95	90	
With S1	8	28	
With Gemcitabine+S1	5	13	
With Other agents[a]	4	7	
Radiation alone	4	7	
Duration[b] (days), median (range)	99.5 (56–278)	82 (46–391)	<0.0001
Chemotherapy, *n* (%)	65 (11)	50 (13)	0.43
Agents provided			
Gemcitabine	17	22	
S1	5	2	
Gemcitabine+S1	26	24	
Gemcitabine+Other agents[c]	17	2	
Duration[b] (days), median (range)	28 (13–138)	81.5 (16–137)	0.0029
No record	6	13	

[a] Other agents was 5-FU + CDDP + MMC ($n = 11$)

[b] Duration represents the days from the start of neoadjuvant therapy to operation

[c] Other agents included 5-FU ($n = 18$) and CDDP ($n = 1$)

Table 3 Adverse events during neoadjuvant therapy

	Grade 1–4 (%)			Grade 3–4 (%)		
	Chemotherapy	Radiotherapy	*P*-value	Chemotherapy	Radiotherapy	*P*-value
Neutrocytopenia	58.8	65.4	0.30	33.8	20.0	0.0164
Leukocytopenia	53.8	75.5	0.0003	16.3	36.7	0.0007
Anemia	28.2	61.5	<0.0001	1.4	3.9	0.45
Thrombocytopenia	26.7	35.7	0.16	4.0	2.8	0.70
Fatigue	20.8	33.2	0.048	0.0	0.0	>0.99
Allergy	13.7	9.7	0.37	0.0	1.1	>0.99
Nausea/Vomiting	9.7	29.6	0.0006	1.4	2.2	>0.99
Liver dysfunction	3.1	0.4	0.055	0.0	0.0	>0.99
Pigmentation	2.3	1.8	0.70	0.0	0.0	>0.99
Anorexia	1.7	8.6	0.010	0.9	2.2	0.67
Cholangitis/Cholecystitis	0.8	2.5	0.43	0.0	2.1	0.17
Pneumonitis	0.0	2.5	0.094	0.0	1.3	0.56
Body weight loss	0.0	2.1	0.17	0.0	0.0	>0.99
Other[a]	6.1	2.5	0.93	0.0	0.0	>0.99

[a] Other non-hematological adverse events included thrombosis, peptic ulcer, oral mucositis, renal dysfunction, constipation

leukocytopenia occurred in more than half of the patients who received neoadjuvant therapy. Any grade leukocytopenia ($P = 0.0003$), anemia ($P < 0.0001$), fatigue ($P = 0.048$), nausea/vomiting ($P = 0.0006$), and anorexia ($P = 0.01$) were significantly more frequent in patients receiving chemoradiotherapy than systemic chemotherapy. Grade 3/4 neutropenia was significantly more frequent in patients receiving chemotherapy ($P = 0.0164$), whereas grade 3/4 leukocytopenia was significantly more frequent in patients receiving chemoradiotherapy ($P = 0.0007$). There were significant differences in any other AE.

Radiological tumor response to neoadjuvant therapy was assessed by tumor reduction rate, shown by waterfall chart analysis (Fig. 2). The median tumor reduction rate was 6.3% (range, –45.2–93.9%). According to Response Evaluation Criteria In Solid Tumors (RECIST) guidelines, 16% of patients showed a partial response, 80% had stable disease, and 4% had progressive disease (PD); none had a complete response to neoadjuvant therapy. Responses to neoadjuvant therapy were similar in patients with resectable and borderline resectable tumors ($P = 0.14$).

Resectability

Of the 388 patients who received neoadjuvant treatment, 25, including eight with resectable and 17 with borderline resectable tumors, did not undergo surgery, including 21 (84%) with PD and one with an AE during preoperative treatment. Of the 582 patients with resectable disease, 397 were scheduled for surgery-first, and, of these, 375 (94.5%) underwent resection. Similarly, of the 185 patients with resectable disease who received neoadjuvant therapy, 171 (92.4%) underwent resection ($P = 0.34$). R0 resection was performed on 305 patients in the surgery-first group and 164 in the neoadjuvant group. The R0 rate was significantly higher in the neoadjuvant than in the surgery-first group, both by on-treatment ($P < 0.0001$) and intention to treat ($P = 0.0003$) analysis (Table 4a). Of the 389 patients with borderline resectable disease, 186 were scheduled to undergo surgery first, and, of these, 156 (83.9%) underwent resection. Similarly, of the 203 patients with borderline resectable disease who received neoadjuvant treatment, 156 (77.8%) underwent resection ($P = 0.16$). Curability assessment showed no significant differences between the two groups, both by on-treatment and intention-to-treat analysis (Table 4b).

Perioperative outcomes

Perioperative morbidity and mortality were evaluated in the 870 patients who underwent pancreatic resection, after excluding the 76 patients who underwent exploratory or bypass surgery. Of these 870 patients, 16 (1.8%) died. In the 546 patients with resectable tumors, there were no significant differences between the neoadjuvant and surgery-first groups in the proportions that underwent various operative procedures or combined resection of major vessels. Operation time was significantly longer ($P = 0.0001$) and blood loss was significantly greater ($P = 0.0059$) in the neoadjuvant than in the surgery-first group. There were six operative deaths (1.6%) in the surgery-first group and one (0.6%) in the neoadjuvant group ($P = 0.44$). Median postoperative hospital stay was significantly longer

Table 4 Resection and R0-resection rate: (**a**) Resectable ($n = 582$) and (**b**) Borderline ($n = 389$)

Group	Surgery first	Neoadjuvant	*P*-value
(a) Resectable ($n = 582$)			
Total cohort, *n*	397	185	–
Resection, *n*	375	171	0.34
Resection rate	94.5%	92.4%	
R0 resection, *n*	305	164	
R0 rate by on-treatment analysis[a]	81.3%	95.9%	<0.0001
R0 rate by intention-to-treat analysis[b]	76.8%	88.6%	0.0003
(b) Borderline ($n = 389$)			
Total cohort	186	203	–
Resection	156	158	
Resection rate	83.9%	77.8%	0.16
R0 resection	118	123	
R0 rate by on-treatment analysis[a]	75.6%	77.8%	0.57
R0 rate by intention-to-treat analysis[b]	63.4%	60.6%	0.61

[a] R0 rate by on-treatment analysis was R0 resection per all resected cases with a record of residual tumor assessment

[b] R0 rate by intention-to-treat analysis was R0 resection per total cases with a record of residual tumor assessment including non-resected and non-operated cases as R2 resection

($P = 0.0020$), and morbidity rate was slightly but not significantly higher ($P = 0.084$) in the neoadjuvant than in the surgery-first group. There were no significant differences in specific postoperative complications, including pancreatic fistula and delayed gastric emptying, as well as in rates of severe complications and reoperation (Table 5).

Of the 314 patients who underwent resection for borderline resectable tumors, those who received neoadjuvant treatment were significantly more likely to undergo resection of the pancreas head ($P = 0.0026$) and portal vein ($P = 0.0018$) than those who underwent surgery first. Operation time was significantly longer in the neoadjuvant than in the surgery-first group ($P = 0.0005$), but there were no between group differences in blood loss ($P = 0.16$), mortality ($P = 0.17$), and hospital stay ($P = 0.50$) (Table 6). Morbidity tended to be less frequent in the neoadjuvant group than in the surgery-first group ($P = 0.057$). In contrast to patients with resectable tumors, the postoperative pancreatic fistula (POPF) rates in patients with borderline resectable tumors were significantly lower in the neoadjuvant group than in the surgery-first group, both for all grades ($P = 0.022$) and grade B/C ($P = 0.015$). Fluid collection was significantly more frequent in the neoadjuvant than in the surgery-first group ($P = 0.016$). Other specific complications and their severity were similar in these two groups (Table 6). In the resectable group with neoadjuvant therapy followed by resection, the proportion of delayed gastric emptying (DGE) in chemoradiotherapy was significantly higher than that in chemotherapy (21.6% vs. 10.1%, $P = 0.0015$). The proportion of other postoperative complications as well as severity of complications and reoperation listed in Table 5 was similar in both treatment modalities. In the borderline group with neoadjuvant therapy followed by resection, the proportion of grade B/C POPF in chemotherapy was slightly, but not statistically significant, higher than that in chemoradiotherapy (10.5% vs. 4.8%, $P = 0.092$). The proportion of other postoperative complications as well as severity of complications and reoperation listed in Table 6 was similar in both treatment modalities.

Histological staging

Table 7 shows a univariate comparison of histological staging according to the American Joint Committee on Cancer (AJCC). Of patients with resectable tumors, those who received neoadjuvant therapy had a lower T grade of the primary tumor than those who underwent surgery first ($P = 0.033$). Moreover, the percentage of patients with lymph node-positive tumors was significantly lower in the neoadjuvant than in the surgery-first group (30.6% vs. 55.2%, $P < 0.0001$), resulting in a significantly lower stage in the former ($P < 0.0001$). In patients with borderline resectable tumors, those who received neoadjuvant treatment had a significantly lower grade of the primary tumor ($P = 0.042$), a significantly lower rate of node-positive tumors (44.3% vs. 74.8%, $P < 0.0001$), and a significantly lower tumor stage ($P < 0.0001$).

Discussion

This survey clarified the feasibility, efficacy, and perioperative outcomes including resectability following

Table 5 Peri-operative outcome in resecteable group

Group	Surgery first	Neoadjuvant	*P*-value
Resection, *n*	375	171	–
PD, *n* (%)	236 (62.9)	111 (64.9)	0.66
DP, *n* (%)	126 (33.6)	52 (30.4)	0.46
TP, *n* (%)	12 (3.2)	7 (4.1)	0.60
PV resection, *n* (%)	71 (18.9)	37 (21.6)	0.46
Arterial resection, *n* (%)	4 (1.1)	4 (2.3)	0.27
Operative time (ml), median (range)	404 (141–829)	470 (157–1,021)	0.0001
Blood loss (ml), median (range)	872 (50–16,422)	1,088 (55–12,925)	0.0059
Blood transfusion (U), median (range)	2 (0–52)	2 (0–16)	0.65
Postoperative hospital stay (day), median (range)	31 (7–167)	36 (8–115)	0.0020
Morbidity, *n* (%)	194 (51.7)	102 (59.7)	0.084
POPF (all grade), *n* (%)	90 (24.0)	35 (20.5)	0.36
POPF (grade B/C), *n* (%)	43 (11.5)	20 (11.7)	0.94
DGE	40 (10.7)	27 (15.8)	0.10
Hemorrhage	16 (4.3)	7 (4.1)	0.93
Abscess	38 (10.1)	19 (11.1)	0.73
Wound infection	30 (8.0)	17 (9.9)	0.46
Leakage[a]	5 (1.3)	6 (3.5)	0.11
Pneumonitis	8 (2.1)	3 (1.8)	>0.99
Thrombosis	3 (0.8)	2 (1.2)	0.65
Cardiac disease	4 (1.0)	0 (0.0)	0.31
Brain	0 (0.0)	1 (0.6)	0.31
Fluid collection/	16 (4.3)	4 (2.3)	0.33
Hepatic disorder	4 (1.1)	4 (2.3)	0.27
Catheter infection	3 (0.8)	2 (1.2)	0.65
Ileus	4 (1.1)	1 (0.6)	>0.99
Cholangitis	4 (1.1)	0 (0.0)	0.31
Diarrhea/enteritis	10 (2.7)	6 (3.5)	0.59
DIC	2 (0.5)	1 (0.6)	>0.99
UTI	1 (0.3)	1 (0.6)	0.53
Renal disorder	2 (0.5)	0 (0.0)	>0.99
Anaphylaxis	1 (0.3)	1 (0.6)	0.53
Sepsis	1 (0.3)	0 (0.0)	>0.99
Splenic infarction	1 (0.3)	1 (0.6)	0.53
Peptic ulcer	1 (0.3)	0 (0.0)	>0.99
Herpes Zoster	2 (0.5)	0 (0.0)	>0.99
Portal vein trouble	1 (0.3)	0 (0.0)	>0.99
Severe complication (Grade IIIa–V), *n* (%)	58 (15.8)	23 (13.9)	0.59
Reoperation	9 (2.4)	7 (4.1)	0.29
Mortality, *n* (%)	6 (1.6)	1 (0.6)	0.44

[a] Leakage includes anastomosis insufficiency except for pancreatic fistula

neoadjuvant therapy in patients with pancreatic cancer. Adjuvant chemotherapy with gemcitabine is a standard therapy following resection for pancreatic cancer and significantly enhances recurrence-free and overall survival compared with surgery alone, with a median overall survival of almost 2 years after surgery [3–5]. However, this approach of surgery followed by adjuvant therapy cannot be offered to a significant proportion of patients with pancreatic cancer because of risks of surgical morbidity and the presence of unresectable disease at laparotomy. In contrast, almost all patients can receive neoadjuvant therapy before surgery [17, 18].

A major concern in treating these patients with neoadjuvant therapy is the risks of operative morbidity and

Table 6 Peri-operative outcome in borderline group

Group	Surgery first	Neoadjuvant	*P*-value
Resection, *n*	156	158	–
PD, *n* (%)	95 (60.9)	121 (76.6)	0.0026
DP, *n* (%)	51 (32.7)	31 (19.6)	0.0081
TP, *n* (%)	9 (5.8)	6 (3.8)	0.44
PV resection, *n* (%)	84 (53.9)	112 (70.9)	0.0018
Arterial resection, *n* (%)	13 (8.3)	10 (6.3)	0.50
Operative time (ml), median (range)	496 (161–1,221)	567 (190–1,160)	0.0005
Blood loss (ml), median (range)	1,137 (20–16,201)	1,400 (60–8,422)	0.16
Blood transfusion (U), median (range)	4 (0–54)	4 (0–18)	0.51
Postoperative hospital stay (day), median (range)	30 (7–397)	31 (8–124)	0.50
Morbidity, *n* (%)	93 (50.0)	82 (40.4)	0.057
POPF (all grade), *n* (%)	34 (18.3)	16 (7.9)	0.0022
POPF (gradeB/C), *n* (%)	19 (10.2)	8 (3.9)	0.015
DGE	24 (12.9)	20 (9.9)	0.34
Hemorrhage	3 (1.6)	4 (2.0)	0.55
Abscess	16 (8.6)	17 (6.4)	0.41
Wound infection	18 (9.7)	20 (9.9)	0.95
Leak[a]	8 (4.3)	3 (1.5)	0.13
Pneumonitis	2 (1.1)	4 (2.0)	0.69
Thrombosis	1 (0.5)	1 (0.5)	1.0
Cardiac disease	0 (0.0)	2 (1.0)	0.50
Brain	2 (1.1)	1 (0.5)	0.61
Fluid collection/	5 (2.7)	17 (8.4)	0.016
Hepatic disorder	3 (1.6)	5 (2.5)	0.73
Catheter infection	1 (0.5)	2 (1.0)	0.53
Ileus	1 (0.5)	0 (0.0)	0.48
Cholangitis	2 (1.1)	2 (1.0)	0.65
Diarrhea/enteritis	4 (2.2)	9 (4.4)	0.26
DIC	0 (0.0)	0 (0.0)	–
UTI	0 (0.0)	0 (0.0)	–
Renal disorder	0 (0.0)	0 (0.0)	–
Anaphylaxis	0 (0.0)	0 (0.0)	–
Splenic infarction	0 (0.0)	0 (0.0)	–
Peptic ulcer	1 (0.5)	1 (0.5)	1.0
Herpes Zoster	0 (0.0)	0 (0.0)	–
Portal vein trouble	1 (0.5)	1 (0.5)	1.0
Severe complication (Grade IIIa–V), *n* (%)	22 (14.5)	21 (13.7)	0.85
Reoperation	6 (3.9)	6 (3.8)	0.98
Mortality, *n* (%)	2 (1.3)	7 (4.4)	0.17

[a] Leakage includes anastomosis insufficiency except for pancreatic fistula

mortality. Although several small prospective studies have demonstrated the feasibility of this approach [10, 11, 19], this has not been confirmed because of the small sample sizes. Several nationwide surveys [20, 21] and systematic reviews and meta-analyses [22, 23] indicated that this strategy was feasible in larger numbers of patients, but could not quantify the data. Only one systematic review showed the rate of surgical morbidity and mortality after neoadjuvant therapy [24]. We found that neoadjuvant treatment did not significantly increase perioperative mortality and morbidity rates, including pancreatic fistula and delayed gastric emptying, indicating that neoadjuvant treatment was a feasible strategy in patients with pancreatic cancer. Neoadjuvant therapy, however, resulted in significantly longer operation times and postoperative hospital stay, as well as higher rates of grade 3/4 hematological toxicities. Nevertheless, these preoperative toxicities were manageable, with <0.5% of patients becoming ineligible for surgery.

Table 7 Peri-operative outcome in resecteable group: (**a**) Resectable and (**b**) Borderline

		Surgery first	Neoadjuvant	*P*-value
(a) Resectable				
T	0	1 (0.3)	2 (1.2)	0.033
	1	33 (8.8)	28 (16.5)	
	2	35 (9.3)	13 (7.7)	
	3	304 (81.1)	124 (72.9)	
	4	2 (0.5)	3 (1.8)	
N	0	168 (44.8)	118 (69.4)	<0.0001
	1	207 (55.2)	52 (30.6)	
M	0	354 (94.4)	160 (94.1)	0.895
	1	21 (5.6)	10 (5.9)	
Stage	0	0 (0)	2 (0.4)	<0.0001
	IA	28 (7.5)	24 (14.1)	
	IB	28 (7.5)	10 (5.9)	
	IIA	110 (29.3)	81 (47.7)	
	IIB	186 (49.6)	40 (23.5)	
	III	2 (0.5)	3 (1.7)	
	IV	21 (5.6)	10 (5.9)	
(b) Borderline				
T	0	2 (1.3)	1 (0.6)	0.042
	1	2 (1.3)	12 (7.6)	
	2	4 (2.6)	9 (5.7)	
	3	140 (90.3)	129 (82.2)	
	4	7 (4.5)	6 (3.8)	
N	0	39 (25.2)	88 (55.7)	<0.0001
	1	116 (74.8)	70 (44.3)	
M	0	132 (85.2)	143 (90.5)	0.895
	1	23 (14.8)	15 (9.5)	
Stage	0	0 (0.0)	1 (0.6)	<0.0001
	IA	1 (0.7)	10 (6.3)	
	IB	2 (1.3)	5 (3.2)	
	IIA	32 (20.7)	64 (40.5)	
	IIB	91 (58.7)	58 (36.7)	
	III	6 (3.9)	5 (3.2)	
	IV	23 (14.8)	15 (9.5)	

Another concern associated with the neoadjuvant strategy is a possible decrease in tumor resectability due to tumor progression during preoperative treatment. A meta-analysis showed that, of patients with resectable tumors, 73.6% to 82.9% remained resectable after neoadjuvant therapy [17, 24], findings similar to those in patients scheduled for primary resection and adjuvant therapy. We found that neoadjuvant therapy did not decrease tumor resectability, both in patients with resectable and borderline resectable pancreatic cancers. Intention-to-treat analysis showed that, in resectable tumors, the curability (R0 resection rate) was improved after neoadjuvant treatment. Radiologically, 90% of patients who received neoadjuvant therapy showed lack of tumor progression or tumor shrinkage, with only 10% showing tumor progression, suggesting that neoadjuvant treatment increased the likelihood of curative resection. These advantages of neoadjuvant therapy, however, were not observed in patients with borderline resectable disease, and resectability and R0 resectability were similar in the neoadjuvant and surgery-first groups. The incidence of nodal involvement was significantly lower in the neoadjuvant than in the surgery-first group. Neoadjuvant therapy has been reported to reduce the number of lymph node metastases [25, 26], suggesting that the main effect of neoadjuvant therapy is to reduce peripancreatic lymph node positivity rather than the size of primary tumors. Since nodal involvement is one of the most significant predictors of patient survival [27, 28], neoadjuvant therapy may have a survival benefit following resection of pancreatic cancer.

Although the number of patients receiving neoadjuvant therapy is the largest to date, questionnaire surveys have limitations. Data were collected from the various treatment centers retrospectively, not prospectively. In addition, there was significant inter-center heterogeneity in eligibility criteria for neoadjuvant treatment, neoadjuvant regimens, radiologic and intraoperative indications for resection, and postoperative therapy regimens. This heterogeneity may have introduced selection biases, preventing definite conclusions. Prospectively designed trials with adequate numbers of patients are required to determine the feasibility and efficacy of neoadjuvant treatment in patients with pancreatic cancer. This survey analyzing the effects of neoadjuvant treatment on resectablity and perioperative outcomes in patients with pancreatic cancer could not determine the impact of treatment on survival. However, several studies have reported that neoadjuvant therapy had survival benefits in patients with resectable or borderline resectable pancreatic cancer [11, 17, 21, 22, 24]. These suggest the need for prospective randomized studies to clarify the effects on survival of neoadjuvant therapy compared with the standard surgery-first strategy, in patients with pancreatic cancer [12, 18]. In conclusion neoadjuvant therapy may not increase the mortality and morbidity rates, and may be able to increase the chance for curative resection especially against resectable tumor.

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Conflict of interest None declared.

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Antitumor immune response of dendritic cells (DCs) expressing tumor-associated antigens derived from induced pluripotent stem cells: In comparison to bone marrow-derived DCs

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It is generally accepted that the difficulty in obtaining a sufficient number of functional dendritic cells (DCs) is a serious problem in DC-based immunotherapy. Therefore, we used the induced pluripotent stem (iPS) cell-derived DCs (iPSDCs). If the therapeutic efficacy of iPSDCs is equivalent to that of bone marrow-derived DCs (BMDCs), then the aforementioned problems may be solved. In our study, we induced iPSDCs from iPS cells and examined the capacity for maturation of iPSDCs compared to that of BMDCs in addition to the capacity for migration of iPSDCs to regional lymph nodes. We adenovirally transduced the hgp100 gene, natural tumor antigens, into DCs and immunized mice once with the genetically modified DCs. The cytotoxic activity of CD8 (+) cytotoxic T lymphocytes (CTLs) was assayed using a ^{51}Cr-release assay. The therapeutic efficacy of the vaccination was examined in a subcutaneous tumor model. Our results showed that iPSDCs have an equal capacity to BMDCs in terms of maturation and migration. Furthermore, hgp100-specific CTLs were generated in mice immunized with genetically modified iPSDCs. These CTLs exhibited as high a level of cytotoxicity against B16 cells as BMDCs. Moreover, vaccination with the genetically modified iPSDCs achieved as high a level of therapeutic efficacy as vaccination with BMDCs. Our study clarified experimentally that genetically modified iPSDCs have an equal capacity to BMDCs in terms of tumor-associated antigen-specific therapeutic antitumor immunity. This vaccination strategy may therefore be useful for future clinical application as a cancer vaccine.

Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in the initiation of antitumor immune responses.[1–3] Many cancer patients worldwide have been treated with cancer vaccine therapy using DCs. Our previous Phase I clinical trial of cancer vaccine therapy using carcinoembryonic antigen peptide-pulsed DCs found the clinical effects of this therapy to be insufficient.[4] Therefore, we next used a gene-based vaccination strategy that used DCs adenovirally transduced with the entire tumor-associated antigen (TAA) gene. We demonstrated that DCs adenovirally transduced with the TAA gene are effective in inducing TAA-specific cytotoxic T lymphocytes (CTLs) and that these cells elicit potent antitumor responses, especially in gastrointestinal solid tumors.[5–8] These findings suggest that this strategy would be useful in clinical application as a cancer vaccine in patients with gastrointestinal tumors, and clinical trials evaluating such applications are now under consideration.

The issue of limited cells is a serious obstacle for DC vaccine therapy. DCs created for clinical use are generated from the peripheral blood monocytes of patients. Therefore, a large amount of blood must be collected or leukapheresis must be performed, which is both expensive and time-consuming. An additional problem is that the number of DCs is reduced in the peripheral blood and the function of DCs is impaired in cancer patients.[9,10] Therefore, obtaining a sufficient number of functional DCs remains a serious problem for the application of DC vaccine therapy.

Recent studies have revealed that embryonic stem cell-like pluripotent stem cells, known as induced pluripotent stem (iPS) cells, can be generated from murine and human fibroblasts.[11,12] Furthermore, it has been reported that DCs can be successfully derived from murine iPS cells (iPSDCs).[13] If the therapeutic efficacy of iPSDCs is equivalent to that of bone marrow-derived DCs (BMDCs), then the aforementioned problems may be solved. Therefore, in our study, we transduced the TAA gene into iPSDCs and examined whether the genetically modified iPSDCs can induce TAA-specific CTLs as effectively as BMDCs.

In our study, iPSDCs were adenovirally transduced with the entire natural tumor antigen hgp100 gene,[14] and whether vaccination with these genetically engineered iPSDCs can

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What's new?

Dendritic cells (DCs) loaded with tumor antigens are important components of immune-based cancer therapies. However, the limited recovery of bone-marrow derived DCs from the blood of cancer patients is a serious obstacle to the common use of DC-based treatments. Here, the authors present an important alternative. They show that DCs derived from induced pluripotent stem cells (iPSCs) are equal in antigen-presentation and migration properties to bone-marrow derived DCs. In a model using adenoviral transduction of the gene encoding tumor-associated antigen, they demonstrate similar antitumor immune responses elicited by both DC types. This new technique may help overcome the limitations of traditional DC-based therapies and may represent a significant step towards a more effective personalized anticancer medicine.

induce strong therapeutic antitumor immunity equivalent to vaccination with genetically engineered BMDCs was assessed.

Material and Methods

Mice

Female C57BL/6 (H-2^b) mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. Six- to 8-week-old mice were used for the experiments. All animal experiments were performed in accordance with the Japanese Government's Animal Protection and Management Law (No. 105) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (No. 88), as well as the guidelines for animal experiments of Wakayama Medical University. Our study plan was approved by the Committee of Animal Experiments (No. 520) and the Committee of Gene Recombination (No. 23-5) of Wakayama Medical University.

Cell lines

The murine embryonic fibroblast-derived iPS cell line iPS-MEF-Ng-20D-17[15] was provided by the RIKEN BioResource Center (Ibaraki, Japan). The iPS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% embryonic stem screened fetal bovine serum (FBS) (Thermo Scientific, Yokohama, Japan), 2 mM L-glutamine (Thermo Scientific), 100 U/ml of penicillin, 100 mg/ml of streptomycin (Life Technologies Co., Carlsbad, CA), nonessential amino acids (Life Technologies) and 50 μM of 2-mercaptoethanol (2-ME) (Life Technologies) on feeder cell layers of mitomycin C-treated murine SNL76/7 cells (European Collection of Cell Cultures, London, UK).[16] Murine bone marrow stromal cells, OP9,[17] were provided by the RIKEN BioResource Center. The cells were maintained in α-MEM supplemented with 20% FBS and seeded onto gelatin-coated dishes before use as feeder cells. The C57BL/6-derived B16 melanoma cell lines expressing hgp100 were maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. The murine chemically induced colon carcinoma cell line MC38 was maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 2 mM L-glutamine. The murine cell line derived from lymphoma, YAC-1, was purchased from RIKEN BioResource Center and maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 2 mM L-glutamine. Recombinant murine (rm) tumor necrosis factor (TNF)-α and granulocyte macrophage colony stimulating factor (GM-CSF) were purchased from Peprotech (London, UK).

Recombinant adenoviral vectors

The adenoviral vector expressing hgp100, AxCAhgp100,[18] was provided by the RIKEN BioResource Center. The recombinant AxCALacz expressing a *Lacz* reporter gene was generated according to the COS-TPC method, as previously described.[5]

Generation of DCs from bone marrow cells

DCs were obtained from murine bone marrow precursors as previously described.[19] In brief, murine bone marrow cells (2.0×10^6) were cultured in 100-mm dishes in 10 ml of complete medium containing 200 units/ml of rmGM-CSF. The complete medium consisted of RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 50 μM of 2-mercaptoethanol. On Day 3 of culture, another 10 ml of fresh medium containing rmGM-CSF was added to the dishes. On Days 6 and 8, half of the medium was replaced by fresh complete medium containing rmGM-CSF. On Day 10, the nonadherent cells were collected and used as immature BMDCs. Other collected cells were cultured for 2 days in another 10 ml of fresh medium containing 100 units/ml of rmGM-CSF and 500 units/ml of rmTNF-α. On day 12, the nonadherent cells were collected for both a flow cytometric analysis and genetic modification and then were used as mature BMDCs.

Generation of DCs from iPS cells

The procedure for inducing the differentiation of iPS cells into mature DCs is composed of four steps, as previously described.[13] In brief, in Step 1, iPS cells were suspended in α-MEM supplemented with 20% FBS and seeded onto OP9 cell layers in dishes. On Day 7, the cells were collected for Step 2 of the culture. In Step 2, the harvested cells were suspended in α-MEM supplemented with 20% FBS, 1,000 U/ml of rmGM-CSF and 50 μM of 2-ME and plated onto new OP9 cell layers. On Day 14, the floating cells were collected *via* pipetting for Step 3 of the culture. In Step 3, the cells were transferred to bacteriological Petri dishes (Locus, Tokyo, Japan) without feeder cells and cultured in complete medium

containing 1,000 U/ml of rmGM-CSF. On Day 26, the floating cells were collected for a flow cytometric analysis *via* pipetting for Step 4 of the culture and used as immature iPSDCs. In Step 4, after 2 days, the collected cells were cultured in another 10 ml of fresh medium containing 1,000 units/ml of rmGM-CSF and 5,000 units/ml of rmTNF-α. On Day 28, the nonadherent cells were collected for a flow cytometric analysis and genetic modification and used as mature iPSDCs.

Flow cytometric analysis

The phenotypic analysis of the DCs preparations was performed with a FACSCalibur (Becton Dickinson, San Jose, CA) using the Cell Quest Pro software program, as previously described.[5,6] DCs (1.0×10^6 cells) were incubated with specific antibodies in phosphate buffered saline (PBS) for 30 min at 4°C and rinsed twice. The following monoclonal antibodies (mAb) conjugated with fluorescence isothiocyanate (FITC) were used for staining: anti-mouse CD11c (clone HL3), anti-mouse CD80 (clone 16-10A1), anti-mouse CD86 (clone GL-1), anti-mouse MHC class II ($I\text{-}A^b$) molecules (clone 25-9-17) (all from BD Pharmingen, San Jose, CA), anti-mouse CCR7 (ImmunoDetect, Hardwick, MA) and FITC-conjugated anti-goat IgG polyclonal antibodies (Vector Laboratories, Burlingame, CA). Intracellular staining with anti-melanoma (hgp100) polyclonal antibodies (Acris Antibodies, Herford, Germany) and FITC-conjugated anti-goat IgG polyclonal antibodies (Vector Laboratories) was performed using a Fixation and Permeabilization Solution Kit (BD Biosciences, San Jose, CA).

Adenoviral vector-mediated gene transfer into DCs

Mature DCs were transduced with each recombinant adenoviral vector using a centrifugal method, as previously described.[5,6] Briefly, DCs were mixed with adenoviral vectors at 100 multiplicities of infection (MOI) and then centrifuged at 2,000*g* at 37°C for 2 hr. Our previous studies showed that the optimal MOI for AxCAhgp100 and AxCALacz are 100.[5,6] Therefore, the MOI values of these vectors were fixed at 100 in our study. The DCs were then washed twice with PBS and cultured in complete medium containing 200 units/ml of rmGM-CSF for 48 hr and used as genetically modified DCs, and the hgp100 expression of each DC was quantified using intracellular staining flow cytometry. The abbreviations for the vectors are: BMDCs-hgp100, BMDCs transduced with AxCAhgp100; and iPSDCs-hgp100, iPSDCs transduced with AxCAhgp100.

Assays for cytokine secretion

Genetically modified DCs were seeded at a concentration of 5.0×10^5 cells/well and cultured on a 48-well plate for 48 hr. The supernatants were then harvested, and the murine IL-12 (p70) and interferon (IFN)-γ levels were measured using an mIL-12 (p70) ELISA kit and an mIFN-γ ELISA kit, respectively (Thermo Scientific, Waltham, MA), as previously described.[6]

Trafficking study of subcutaneously injected DCs

We examined whether the iPSDCs were able to migrate to regional lymph nodes as well as BMDCs, following the method described previously.[5] DCs (BMDCs or iPSDCs) were labeled with the GREEN fluorescent 15 dye PKH67 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The cells were washed and incubated with PKH67 staining solution for 5 min. Complete medium containing 10% FBS was added to the cells, and unbound PKH67 was removed using extensive washing with PBS. DCs (1.0×10^6 cells) labeled with PKH67 were injected subcutaneously into the flanks of the mice. After 72 hr, the mice were sacrificed, and the draining inguinal lymph nodes were removed. Some of the harvested lymph nodes were gently homogenized and suspended in PBS. The suspensions were then subjected to a cell imaging analysis to detect fluorescence-positive cells and counterstaining with DAPI (Life Technologies) within the lymph node preparation. This analysis was performed using Cellomics CellInsight (Thermo Scientific) according to the manufacturer's instructions. The other harvested lymph nodes were frozen with O.C.T. Compound (Sakura Finetek Japan, Tokyo) in liquid nitrogen. Some of the frozen tissue sections were fixed with 10% formalin and counterstained with hematoxylin and eosin. The other harvested lymph nodes were gently homogenized and suspended in PBS. The suspensions were subsequently subjected to a flow cytometric analysis to detect fluorescence-positive cells.

MHC Class I tetramer assay and the induction of antigen-specific CTLs and cytotoxicity

To determine whether the administration of DCs (BMDCs or iPSDCs) transfected with AxCAhgp100 would induce hgp100-specific CTLs, DCs were transfected with AxCAhgp100 and AxCALacz (at 100 MOI), as described above. C57BL/6 mice were immunized once *via* subcutaneous injection in the flank with genetically modified DCs (1.0×10^6 cells) suspended in 200 μl of PBS. On Day 14, the spleens were removed, and some of the *in vivo*-primed splenocytes (8.0×10^6 cells/well) were pooled and cocultured in a six-well plate (in complete medium containing 50 units/ml of rmIL-2 at 4 ml/well). After 5 days of coculture, the collected cells were incubated with $H\text{-}2D^b$ hgp100 Tetramer or $H\text{-}2D^b$ Influenza NP Tetramer (MBL, Nagoya, Japan) for 20 min at 4°C in combination with anti-CD8 mAb with FITC (MBL) according to the manufacturer's instructions. The incubated cells were subjected to a flow cytometric analysis. The remaining *in vivo*-primed splenocytes (8.0×10^6 cells/well) were pooled and cocultured with mitomycin C-treated B16 cells (8.0×10^5 cells/well) in a six-well plate (in complete medium containing 50 units/ml of rmIL-2 at 4 ml/well). After 5 days of coculture, the CD8(+) CTLs were sorted from the *in vivo*-restimulated splenocytes using an autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and assayed in a 4-hr ^{51}Cr-release assay, as previously described.[5,6]

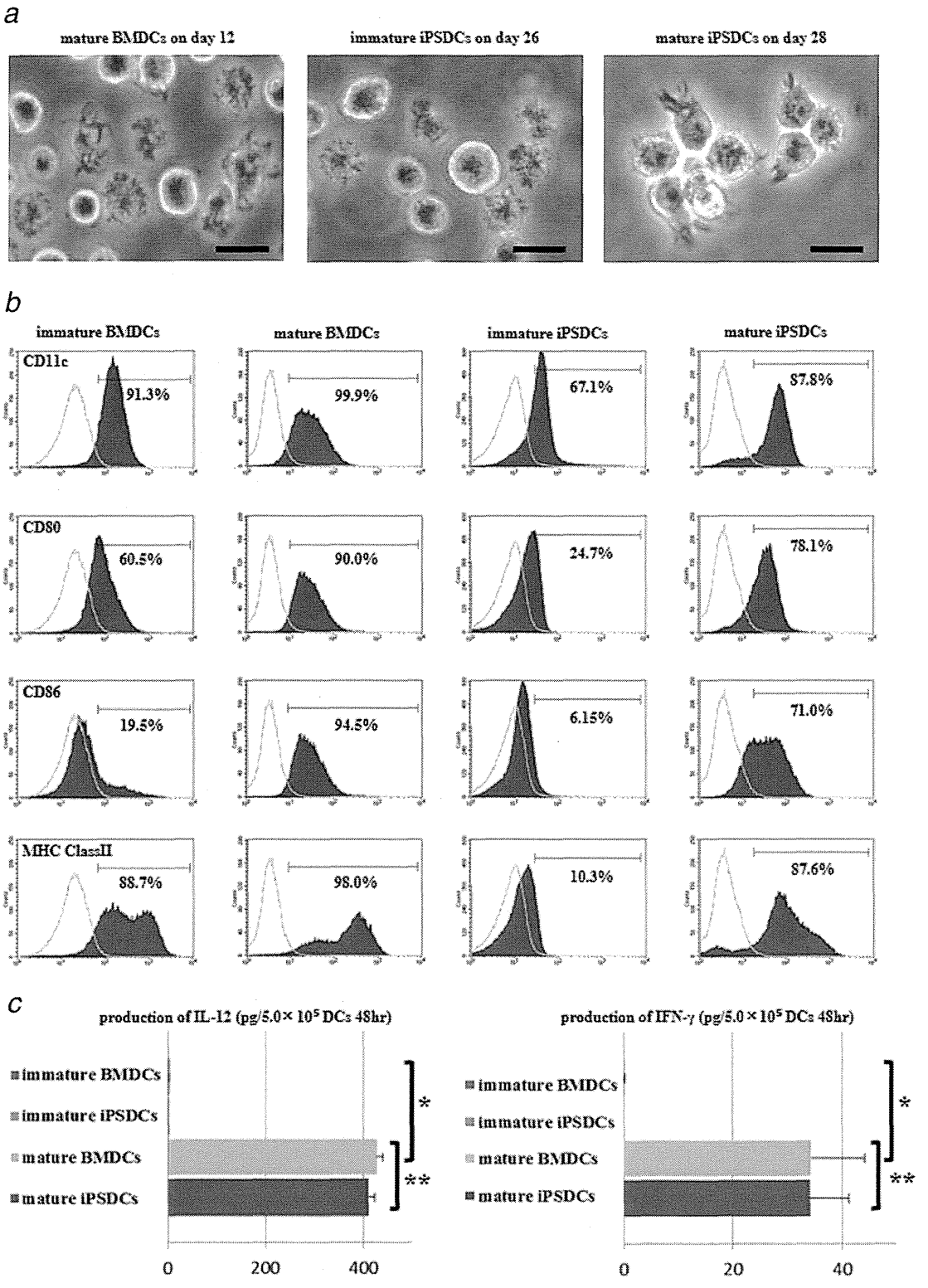

Figure 1. Mature capacity of DCs. (*a*) Morphology of mature BMDCs on Day 12, immature iPSDCs on Day 26 and mature iPSDCs on Day 28. The scale bars represent 20 μm. (*b*) Surface phenotypes of BMDCs and iPSDCs. The staining patterns of specific antibodies (black) and isotype-matched controls (thin lines) are shown in histograms. (*c*) Secretion of IL-12 and IFN-γ from BMDCs and iPSDCs. The results are shown as the mean ± SD ($n = 5$ for each group). *Significantly higher than the immature DCs ($p < 0.0001$). **No significant differences compared to the mature DCs ($p > 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

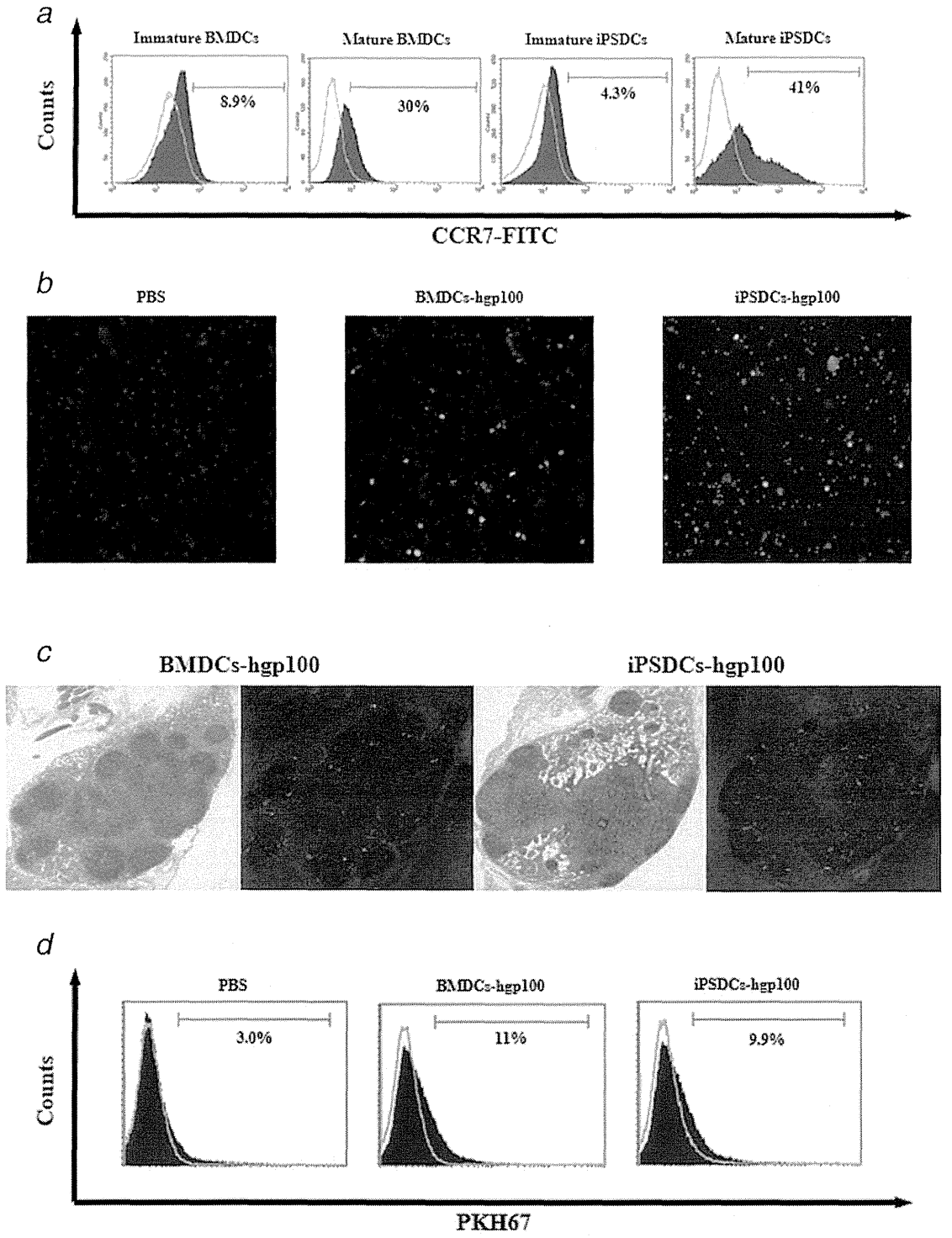

Figure 2. *Migratory capacity of DCs in vivo. (a) BMDCs and iPSDCs were examined for the expression of CCR7 using flow cytometry. The staining patterns of specific antibodies (black) and FITC controls (thin lines) are shown in histograms. (b) Single-cell suspensions obtained from the draining lymph nodes were counterstained with the blue fluorescent dye DAPI in the nuclei and analyzed using a cell imaging analysis. Green fluorescent-positive cells and DCs stained with PKH67 were observed in the BMDCs and iPSDCs (image size: 660.48 μm × 660.48 μm). (c, d) The migratory capacity of DCs in vivo. BMDCs-AxCAhgp100 and iPSDCs-AxCAhgp100 were labeled with the fluorescent dye PKH67 and the DCs and PBS were injected s.c. into the lower abdomen of the mice. After 72 hr, (c) the draining LNs were observed using fluorescence microscopy of the cryostat sections. (d) Single-cell suspensions obtained from the draining LNs were analyzed for labeled cells using flow cytometry. The staining patterns of PKH67 (black) and untreated lymph node-controls (thin lines) are shown in histograms. Histograms represent fluorescence of ungated, total lymph node cell suspensions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

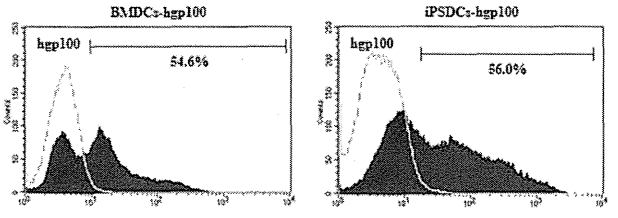

Figure 3. Expression of hgp100 in the genetically modified DCs. The intracellular expression of hgp100 in the genetically modified BMDCs and iPSDCs. DCs were analyzed using intracellular staining flow cytometry. The staining patterns of hgp100 (black) and FITC-matched controls (thin lines) are shown in histograms.

Briefly, B16, MC38 and YAC-1 were used as target cells. The target cells, labeled with $Na_2{}^{51}CrO_4$, were incubated in triplicate with effector cells at various E/T ratios at 37°C in a 5% CO_2 atmosphere for 4 hr. The supernatant was harvested, and the level of radioactivity was counted using a gamma counter. The maximum amount of ^{51}Cr incorporated in the supernatant was determined by adding 1 N HCl to the target cells. The percentage of cytotoxicity was calculated as follows: percentage of lysis = [(sample cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100. The lymph node cells were removed and the *in vivo*-primed lymph node cells were restimulated with mitomycin C-treated B16 cells in the same way as described above. After 5 days of coculture, the *in vivo*-restimulated lymph node cells were assayed in a 4-hr ^{51}Cr-release assay with B16 and MC38 as target cells, as described above.

Experimental design of in vivo tumor therapy for the subcutaneous tumor model

To assess whether pre-existing subcutaneous tumors could be suppressed following immunization with DCs (BMDCs or iPSDCs) genetically modified to express hgp100, C57BL/6 mice were immunized *via* subcutaneous injection in the right flank with B16 cells (1.0×10^6 cells), as previously described.[5,6] On Day 5, the tumor-bearing mice ($n = 5$ mice/group) were treated with subcutaneous injections in the opposite flank with genetically modified DCs (1.0×10^6 cells). The volume of the subcutaneous tumors was estimated every 2 or 3 days using the following formula: (short diameter)^2 × long diameter × 0.52.

Statistical analysis

The SPSS software program ver. 18.0 (SPSS, Chicago, IL) was used for all statistical analyses. Quantitative results were expressed as the mean ± SD. Two-tailed Student's *t*-test was used to determine the statistical significance of differences, and a *p*-value of < 0.05 was considered to be significant.

Results

Generation of DCs from iPS cells

Undifferentiated iPS cells were maintained on the feeder layers of SNL cells. To initiate differentiation, the iPS cells were transferred onto OP9 feeder layers. On Day 3, mesodermally differentiated flat colonies appeared. On Day 7, most of the colonies exhibited a differentiated morphology, and the cells were harvested. Subsequently, the cells were transferred onto new OP9 feeder layers and cultured with rmGM-CSF to start Step 2. In Step 2, homogenous small cells resembling primitive hematopoietic stem cells appeared. The iPS cell-derived round cells gradually increased and became morphologically heterologous. Because the addition of rmGM-CSF was essential for the proliferation of the cells, we changed the medium every 2 or 3 days in Step 2. On Day 14, the floating or loosely adherent cells were recovered *via* pipetting. At the beginning of Step 3, we transferred the cells into bacteriological Petri dishes without feeder cells. On Day 26, most of the floating cells, that is, the immature iPSDCs, exhibited an irregular shape with areas of protrusion. The cells were then cultured with 1,000 units/ml of rmGM-CSF and 5,000 units/ml of rmTNF-α for 2 days in Step 4. Finally, on Day 28, the mature iPSDCs were collected and found to be morphologically similar to mature BMDCs (Fig. 1*a*). The yield of differentiation cells was 440 times the cell number in Step 1, 1.35 times the cell number in Step 2 and 1.05 times the cell number in Step 3. Subsequently, the total yield was more than 600 times from the iPS cells to the iPSDCs.

Expressions of DC surface markers

A flow cytometric analysis demonstrated that the immature iPSDCs exhibited high levels of the cell surface expression of CD11c only. In the mature iPSDCs, however, high levels of the cell surface expression of CD11c, CD80, CD86 and MHC Class II were noted. All cell surface expression ratios in the mature iPSDCs were higher than those observed in the immature iPSDCs, as was also the case in the BMDCs (Fig. 1*b*). All experiments were performed five times to confirm the reproducibility of the results, and similar results were obtained each time.

Secretion of IL-12 and IFN-γ from DCs

Neither immature BMDCs nor iPSDCs secreted IL-12 or IFN-γ. The levels of secretion of IL-12 and IFN-γ by mature BMDCs and iPSDCs were significantly higher than those of the immature DCs (both $p < 0.0001$, Fig. 1*c*). Furthermore, there were no significant differences in IL-12 or IFN-γ secretion between the mature BMDCs and iPSDCs ($n = 5$, $p > 0.05$).

Migratory capacity of DCs in vivo

To evaluate the migratory capacity of the BMDCs and iPSDCs *in vivo*, we compared the *in vivo* function. First, the expression of cell surface CCR7 was analyzed using a flow cytometric analysis. The percentage of positively stained cells was less than 10% in the immature BMDCs and iPSDCs, whereas the percentage in the mature BMDCs and iPSDCs rose to 30% and 41%, respectively (Fig. 2*a*). Next, BMDCs-hgp100 and iPSDCs-hgp100 cells were stably labeled with a

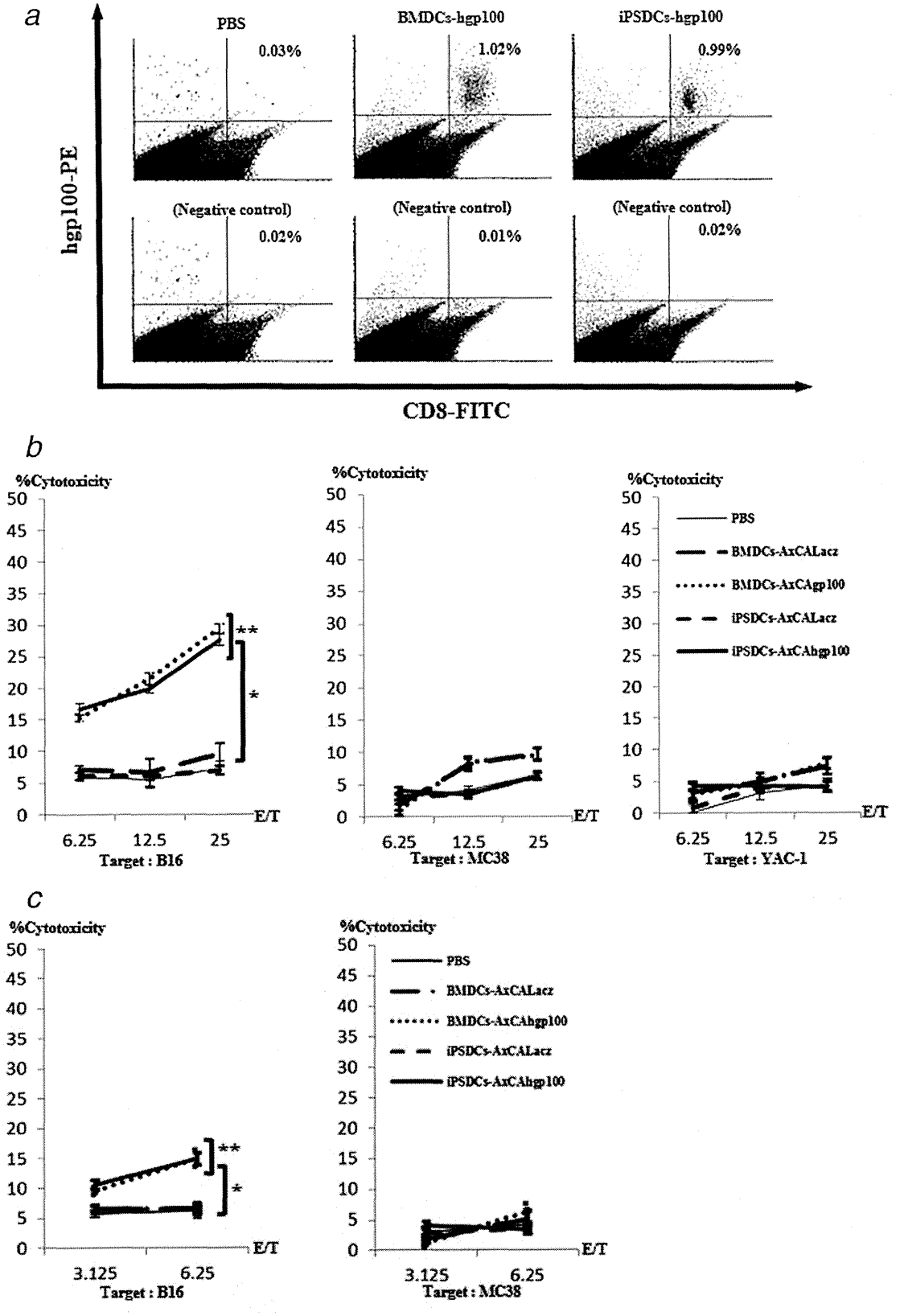
a
PBS
0.03%
BMDCs-hgp100
1.02%
iPSDCs-hgp100
0.99%
(Negative control)
0.02%
(Negative control)
0.01%
(Negative control)
0.02%
hgp100-PE
CD8-FITC
b
%Cytotoxicity
E/T
6.25
12.5
25
Target : B16
Target : MC38
Target : YAC-1
PBS
BMDCs-AxCALacz
BMDCs-AxCAgp100
iPSDCs-AxCALacz
iPSDCs-AxCAhgp100
c
3.125
6.25
Target : B16
Target : MC38

Figure 4.

fluorescent dye (PKH67) and subcutaneously injected into the mice. After 72 hr, single-cell suspensions obtained from the draining lymph nodes were analyzed using cell imaging. There were no DCs with green fluorescent dye in the PBS group (negative control); however, green fluorescent-positive cells were observed in the BMDCs-hgp100 and iPSDCs-hgp100 cells (Fig. 2*b*). Then, the draining lymph nodes were removed and frozen, and the frozen tissue sections counterstained with hematoxylin/eosin were observed using fluorescence microscopy. Among the lymph nodes in the mice injected with BMDCs-hgp100 and iPSDCs-hgp100, green fluorescent-positive cells were observed primarily on the medial side of the cortex (paracortex) and T-cell zone (Fig. 2*c*). According to flow cytometry, the populations of green fluorescent-positive cells in single-cell suspensions obtained from the draining lymph nodes of the mice immunized with PBS, BMDCs-hgp100 and iPSDCs-hgp100 were 3.0% (background fluorescence), 11% and 9.9%, respectively (Fig. 2*d*). All experiments were performed five times to confirm the reproducibility of the results, and similar results were obtained each time.

Expression of hgp100 in the genetically modified DCs

To compare mature BMDCs and iPSDCs in terms of the efficiency of transfecting the hgp100 gene with AxCAhgp100 at 100 MOI, genetically modified DCs were analyzed using intracellular hgp100-staining flow cytometry. The percentage of positively stained cells among the BMDCs and iPSDCs was 54.6% and 56.0%, respectively (Fig. 3).

Cytotoxic activity of CD8(+) CTLs in mice immunized with DCs expressing hgp100

To analyze the capacity of DCs to prime TAA-specific T-cells *in vivo*, a tetramer assay of the CD8 (+) T-cells in the cultured spleen cells isolated from the mice immunized with genetically modified DCs or PBS was performed. The results showed that 0.03%, 1.02% and 0.99% of the CD8 (+) T-cells in the cultured spleen cells isolated from the mice injected with PBS, BMDCs-hgp100 and iPSDCs-hgp100, respectively, were positively stained with the tetramer of hgp100. On the other hand, 0.02%, 0.01% and 0.02%, respectively, of the CD8 (+) T-cells were positively stained with the tetramer of Influenza NP (negative control) (Fig. 4*a*). Next, a ^{51}Cr-release assay was performed to evaluate the cytotoxic activity against the B16 cells of CD8(+) CTLs in the spleens of the mice immunized with genetically modified DCs or PBS. Although the CD8(+) CTLs in the immunized mice exhibited no cytotoxic activity against the MC38 or YAC-1 (NK-sensitive target) cells, they did express significantly higher levels of cytotoxicity against the B16 cells in the mice immunized with genetically modified BMDCs and iPSDCs expressing hgp100 than that observed in the other cells (E/T: 25, $p < 0.0001$). There were no significant differences between the genetically modified DCs expressing hgp100 in terms of the cytotoxic activity against B16 cells (E/T: 25, $p > 0.05$, Fig. 4*b*). Furthermore, to evaluate the cytotoxic activity of the draining lymph nodes cells in the mice immunized with genetically modified DCs or PBS, a ^{51}Cr-release assay was performed. Although the lymph node cells in the immunized mice exhibited no cytotoxic activity against MC38, they did express a significantly higher level of cytotoxicity against the B16 cells in the mice immunized with BMDCs-hgp100 and iPSDCs-hgp100 than that observed in the other cells (E/T: 6.25, $p < 0.05$). There were no significant differences between the genetically modified DCs expressing hgp100 in terms of the cytotoxic activity against B16 cells (E/T: 6.25, $p > 0.05$, Fig. 4*c*).

Therapeutic efficacy of genetically modified DCs in the subcutaneous tumor model

We used treatment schedules in the subcutaneous tumor model to evaluate the therapeutic efficacy of vaccination with genetically modified DCs or PBS against B16 cells. The vaccination with BMDCs-hgp100 and iPSDCs-hgp100 exhibited significantly higher therapeutic efficacy than the other types of vaccination (Day 20, $p < 0.0001$). There were no significant differences between the genetically modified DCs expressing hgp100 in terms of therapeutic efficacy (Day 20, $n = 5$, $p > 0.05$, Fig. 5).

Discussion

Our study demonstrated that iPS cells are capable of differentiating into functional DCs in four steps. In concrete terms,

Figure 4. Cytotoxic activity of CD8(+) CTLs in the mice immunized with DCs expressing hgp100. (*a*) Splenocytes were removed from the mice immunized with genetically modified DCs and cultured with rm IL-2 only. After 5 days, the cultured cells were stained with the tetramers of H-2D^b-hgp100 and H-2D^b-Influenza NP (negative control) in combination with anti-CD8 mAbs and analyzed using flow cytometry. (*b*) The cytotoxic activity of the spleen cells in the mice immunized with genetically modified DCs. The genetically modified DCs were as follows: PBS (——), BMDCs-AxCALacz (— ·), BMDCs-AxCAhgp100 (·····), iPSDCs-AxCALacZ (– –) and iPSDCs-AxCAhgp100 (—). The cytotoxic activity was examined using a ^{51}Cr -release assay. The results are shown as the mean ± SD ($n = 7$ for each group). *Significantly higher cytotoxicity against B16 cells in the mice immunized with genetically modified BMDCs and iPSDCs expressing hgp100 than that observed in the other cells (E/T: 25, $p < 0.0001$). **No significant differences compared to the genetically modified DCs expressing hgp100 (E/T: 25, $p > 0.05$). (*c*) The cytotoxic activity of the lymph node cells in the mice immunized with genetically modified DCs. The genetically modified DCs were as follows: same as above. The cytotoxic activity was examined using a ^{51}Cr-release assay. The results are shown as the mean ± SD ($n = 5$ for each group). *Significantly higher cytotoxicity against B16 cells in the mice immunized with genetically modified BMDCs and iPSDCs expressing hgp100 than that observed in the other cells (E/T: 6.25, $p < 0.05$). **No significant differences compared to the genetically modified DCs expressing hgp100 (E/T: 6.25, $p > 0.05$).

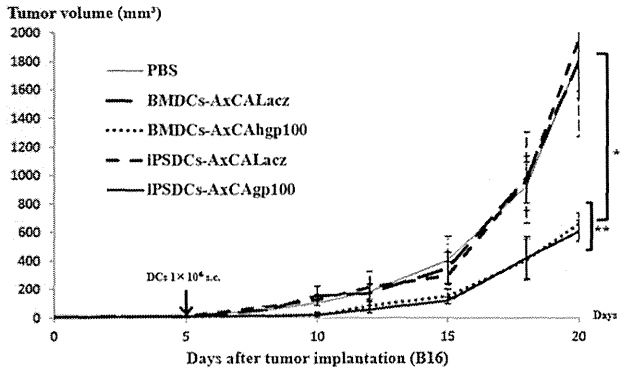

Figure 5. Therapeutic efficacy of genetically modified DCs in the subcutaneous tumor model. Tumor growth suppression in the mice immunized with genetically modified DCs in the subcutaneous tumor model ($n = 5$ for each group). The genetically modified DCs were as follows: PBS (——), BMDCs-AxCALacz (— ·), BMDCs-AxCAhgp100 (·····), iPSDCs-AxCALacZ (– –) and iPSDCs-AxCAhgp100 (—). The results are presented as the mean tumor volume ± SD of the mice that developed tumors in each group. *Significantly higher therapeutic efficacy than that observed in the other cells (Day 20, $p < 0.0001$). **No significant differences compared to the genetically modified DCs expressing hgp100 in terms of therapeutic efficacy (Day 20, $p > 0.05$).

the iPSDCs were fully matured with TNF-α and secreted adequate amounts of IL-12 and IFN-γ, as did the BMDCs. Furthermore, the iPSDCs exhibited an equal migration capacity to that of the BMDCs.

As the generation of iPS cells was first reported, several studies have evaluated organs derived from iPS cells, such as retinal pigment epithelia, platelets and gametes.[20–22] These iPS cell-derived organs demonstrate a similar capacity to naive organs. Senju *et al.*[13] examined the global gene expression profiles of BMDCs and iPSDCs using DNA microarrays. Their results indicated that the gene expression profiles of the two cell types were similar. This similarity in the gene expression likely explains the similar function observed between BMDCs and iPSDCs. Further investigation of this issue is necessary. However, our study experimentally demonstrated that iPSDCs have an equal capacity to BMDCs in terms of maturation and migration. In addition, we demonstrated that injected iPSDCs, as well as BMDCs, can migrate into the nodular paracortex and T-cell zone to interact with T-cells.[1]

The primary advantage of a gene-based vaccination strategy using DCs transduced with the entire TAA gene is that the DCs will present multiple epitopes, including unknown epitopes associated with different MHC Class I molecules, in addition to helper epitopes associated with MHC Class II molecules.[23,24] Our study demonstrated that an adenovirus vector encoding the TAA gene, hgp100, can be used to effectively transfer and express the transgene in iPSDCs. On the other hand, it is generally accepted that iPS cells exhibit high efficiency and simplicity of transgene transfection using electroporation. If iPS cells are stably transfected with the TAA gene before differentiating into DCs, almost 100% of the iPSDCs derived from these iPS cells are expected to express the TAA gene. Therefore, it is a more effective clinical application to transfect the TAA gene into iPS cells before the cells differentiate into DCs. Currently, we seek to induce the differentiation of iPSDCs from iPS cells stably expressing the TAA gene.

We showed that a single vaccination of genetically modified iPSDCs expressing the entire TAA gene elicits a potent therapeutic TAA-specific immunity that results in the suppression of tumor growth. Furthermore, in our study, the vaccination of genetically modified iPSDCs and BMDCs exhibited an equivalent antitumor effect. Several mechanisms are suggested based on the successful induction of antitumor immunity. First, iPSDCs have an equal capacity to BMDCs in terms of maturation and migration, as described above. Therefore, genetically modified iPSDCs expressing TAA at the completely matured state may express more stable MHC Class I TAA-specific peptide complexes, resulting in efficient TAA presentation in the context of costimulatory molecules. Second, iPSDCs have an equal capacity to BMDCs in terms of MHC Class I/II presentation of endogenously expressed antigens. Indeed, our results of the TAA-specific tetramer assay and TAA-specific CTL activity in both splenocytes and regional lymph node suspensions strongly suggest that CTL recognition of cross-reactive MHC Class I epitopes on the TAA molecules can be induced in mice immunized with iPSDCs. Further investigation of this issue is necessary to establish the clinical applications of this strategy.

In conclusion, vaccination with iPSDCs may solve the problems related to the defective function, number and viability of naive DCs obtained from cancer patients. We consider this strategy to be useful for clinical application as a cancer vaccine. Such therapy would include the following four steps: Step 1: culture and proliferate iPS cells generated from the patient's fibroblasts; Step 2: transduce TAA genes into the iPS cells; Step 3: induce these iPS cells into iPSDCs and Step 4: vaccinate the genetically modified iPSDCs expressing the TAA gene as a cancer therapy tailor-made for the patient. There are many issues to be overcome before this vaccination strategy can be applied in clinical practice; however, work is ongoing to meet this goal.

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